SEROLOGICAL COMPARISONS AMONG FOUR CLASSES OF MOLLUSCA [1]

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Serological comparisons of the proteins of organisms are a means of studying the results of biochemical evolution. With the precipitin test the chemical similarities of the proteins of animals can be measured quantitatively and the degrees of relationship thereby estimated. The purpose of this paper is to report on some serological comparisons of the proteins from the four Classes Amphineura, Gastropoda, Pelecypoda, and Cephalopoda in the Phylum Mollusca.

Materials and Methods

Antigens

Table I lists the species of Mollusca from which antigens were obtained. Sera from *Octopus vulgaris* and *Sepia officinalis* were collected at the Stazione Zoologica di Napoli, Naples, Italy in the summer of 1948. Serum from *Busycon caricum* was obtained at the laboratory of the United States Fish and Wildlife Service, Beaufort, North Carolina, in the summer of 1951. The other antigens were collected at the Friday Harbor Marine Laboratory, University of Washington, Friday Harbor, Washington, in the summer of 1952. 'Merthiolate' in a final concentration of 0.01 per cent (1 : 10,000) was added as a preservative at the time of collection.

Antisera

The antisera used in this study were produced in healthy, adult rabbits. Table I shows the schedule of injections followed for the production of these antisera. Multiple series of injections were given to all rabbits except the one which produced the antiserum against the proteins of *Tonicella lineata*. This rabbit received a single series of injections; seven injections were given on alternate days—1.0, 1.5, 2.0, 2.0, 5.0, 5.0, and 5.0 ml. The fifth and sixth were intraperitoneal and the others were intravenous. Each rabbit was given a trial bleeding from the median artery of the ear eight days after the last injection of a series. At the end of the injection schedule those rabbits with good levels of antibody were completely exsanguinated by intracardial puncture after a fast of 18 hours to obtain sera free from dissolved lipids. Whole blood, obtained either by bleeding from the median artery of the ear, or by intracardial puncture, was placed in lusteroid centrifuge tubes, permitted to clot, rimmed, and held at $0 \pm 2°$ C. for 1 to 10 hours. Expressed serum was

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TABLE I

Mollusca used in serological comparisons and injection schedules used to produce antisera

Species	Serum protein gms./100 ml.	Injection schedule	Series of injections
PHYLUM MOLLUSCA			
Class Cephalopoda			
Octopus vulgaris Linnaeus	1.75	1.0, 1.5, 2.0 ml., iv*., alt. days†	4
Sepia officinalis Linnaeus	4.20	1.0, 1.5, 2.0 ml., iv., alt. days	4
Class Gastropoda			
Argobuccinum oregonensis (Redfield)	3.10	1.0, 1.0, 2.0, 2.0 ml., iv., alt. days	2
Busycon caricum (Gmelin)	2.40	1.0 1.5 2.0 ml., iv., alt. days	4
Class Pelecypoda			
Macoma nasuta Conrad	1.27	1.0, 1.5, 2.0, 2.0 ml., iv., alt. days	2
Modiolus modiolus Linnaeus	0.27	1.0, 2.0, 2.0, 2.0 ml., iv., alt. days	2
Pecten hericus Gould	0.65	1.0, 1.0, 2.0, 4.0 ml., iv., alt. days	2
Pecten hindsii (Carpenter)	0.45	1.0, 1.0, 2.0, 4.0 ml., iv., alt. days	2
Pododesmus macroschisma Deshayes	0.47	1.0, 2.0, 2.0, 2.0 ml., iv., alt. days	2
Class Amphineura			
Cryptochiton stelleri Middendorf	2.47	2.0, 1.5, 2.0 ml., iv., alt. days	4
Katharina tunicata Wood	4.80	1.0, 1.5, 2.0 ml., iv., alt. days	4
Mopalia muscosa (Gould)	1.07	1.0, 1.5, 2.0 ml., iv., alt. days	4
Tonicella lineata Wood	0.67	1.0, 1.5, 2.0, 2.0 ml., iv., alt. days, 5.0, 5.0 ml., ip**., 5.0 ml., iv., alt. days	1

*iv = intravenously, **ip = intraperitoneally, † alt. days = alternate days

poured into clean, dry lusteroid centrifuge tubes and centrifuged for 30 minutes at 375 g. The resulting clear serum was decanted, sterilized by passage through Seitz filters, put into sterile serum vials and frozen at − 20° C. until used.

METHODS OF TESTING

Quantitative measures of precipitin reactions were made with the Libby (1938) photronreflectometer, using the methods outlined by Boyden and DeFalco (1943) and Leone (1949). Precipitin tests were performed by adding a constant amount of antiserum to doubling dilutions of antigens. The proteins were diluted with 0.9 per cent saline buffered with *M*/150 phosphate salts. 'Merthiolate' in final concentration of 0.01 per cent (1 : 10,000) was added as a bacteriostatic agent to the saline diluent. Initial dilutions of antigens were made on the basis of the known concentration of protein (Table 1); dilutions ranged from 1 : 62.5 to 1 : 4,096,000. Control readings of antigen and saline, and antiserum and saline, were subtracted from the total turbidity readings. Corrected values for the antigen-antibody precipitates were plotted with the turbidities on the ordinate and the antigen dilutions on the abscissa. Representative curves are shown in Figure 1. A value proportional to the area under each curve was obtained by a summation of the turbidity values (Boyden and DeFalco, 1943). Duplicate tests yield "area" values which are within ± 3 per cent of each other. The "areas" of the homologous and heterologous reactions were used to establish the relationships among the various proteins which were compared. To compute the percentage relationship, the summation of

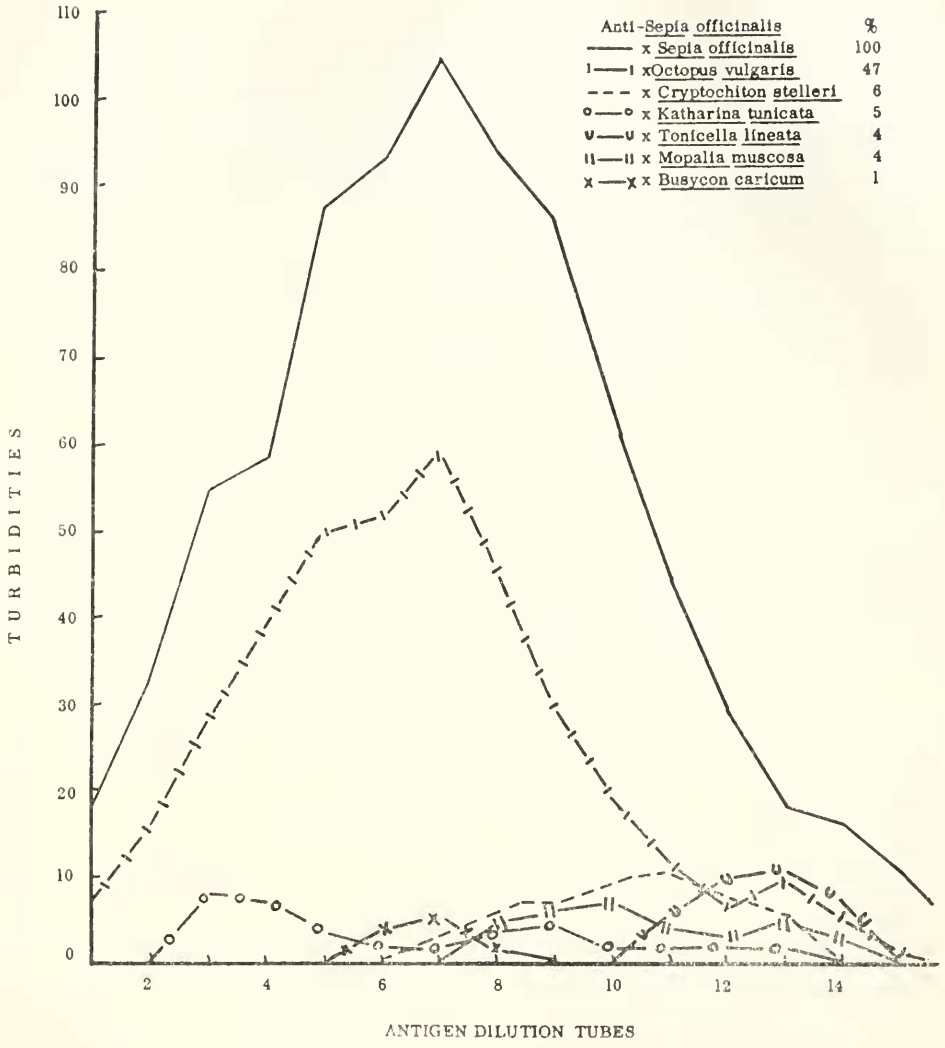

FIGURE 1. Precipitin reaction curves obtained with the antiserum produced against *Sepia officinalis*. Values proportional to the areas under the curves are obtained by summating the turbidity values. Comparisons are made of the "areas" to determine the per cent relationship among the species represented.

the homologous reaction was divided into the summation of the heterologous reaction and the quotient multiplied by 100.

EXPERIMENTAL RESULTS

All antisera produced using the schedules of injections given in Table I had high levels of antibodies. Table II gives a summary of the serological relationships which were calculated from the precipitin tests performed with the various antigens

and antisera. The percentage values in Table II are based on averages of tests done in duplicate.

The antiserum produced against the proteins of *Octopus vulgaris* had the strongest cross-reaction with the proteins of the other cephalopod, *Sepia officinalis*. Proteins from the gastropod *Busycon caricum* were slightly more reactive with this antiserum than were the proteins from the several amphineurans which were tested. The proteins from the pelecypods *Pecten hericus* and *P. hindsii* had no measurable reactions with this antiserum.

The antiserum produced against the proteins of *Sepia officinalis* had the strongest cross-reaction with the proteins of *Octopus vulgaris*. This antiserum did not permit us to distinguish effectively between the gastropods and amphineurans, the proteins of both of which were measurably reactive. The pelecypods *Pecten hericus* and *P. hindsii* had no measurable reactions with the antiserum.

TABLE II*

Per cent relationships among species from four classes of Mollusca

Antigens \ Antisera	*Octopus vulgaris* Cephalopoda	*Sepia officinalis* Cephalopoda	*Argobuccinum oregonensis* Gastropoda		*Busycon caricum* Gastropoda	*Macoma nasuta* Gastropoda	*Modiolus modiolus* Pelecypoda	*Pecten hericus* Pelecypoda	*Pecten hindsii* Pelecypoda	*Pododesmus macroschisma* Pelecypoda	*Cryptochiton stelleri* Amphineura		*Katharina tunicata* Amphineura	*Mopalia muscosa* Amphineura	*Tonicella lineata* Amphineura
Octopus vulgaris	100	47	0	5	1	0	0	0	0	0	1	1	1	1	0
Sepia officinalis	69	100	0	3	0			0	0		2		8	0	0
Argobuccinum oregonensis		0	100	100	33			0	0		0		0		0
Busycon caricum	21	1	58	34	100	0	0	0	0		7	0	2	0	0
Macoma nasuta						100	0			0					
Modiolus modiolus				2		0	100			13		0	0		0
Pecten hericus	0	0	0	3	0	22	4	100	56	0	0	0		0	0
Pecten hindsii	0	0	0		0			74	100			0	0	0	
Pododesmus macroschisma						0	5			100					
Cryptochiton stelleri	16	6	0	0	2			19	6		100	100	79	65	31
Katharina tunicata	14	5	0	1	12	0	0	19	0	0	54	80	100	69	25
Mopalia muscosa	13	4	0								36	65	63	100	26
Tonicella lineata		4	0		0			0			10	20	24	23	100

* Comparative values are in the vertical columns. Experimental error: ± 3 per cent relationship. The columns headed *Argobuccinum* and *Cryptochiton* contain sets of data for two antisera.

The two antisera which were produced against the proteins of the gastropod *Argobuccinum oregonensis* were specific with respect to cross-reactivity with the proteins from representatives of the other Classes in the Phylum Mollusca. Both antisera had strong cross-reactions with the other gastropod, *Busycon caricum*. The less specific of the two antisera was slightly reactive with the proteins from the cephalopods *Octopus* and *Sepia*, the pelecypods *Modiolus* and *Pecten* and the amphineuran *Katharina*. The more specific of the two anti-*Argobuccinum* sera gave measurable cross-reactions only with the proteins of the other gastropod *Busycon*.

The proteins of the other gastropod, *Argobuccinum*, were most reactive with the antiserum to *Busycon caricum*. The reactivity of the proteins of the amphineuran *Katharina tunicata* with this antiserum was strong as compared to the slightly posi-

tive results, and negative results obtained with the proteins of the other amphineurans. Of the cephalopods, only the proteins of *Octopus* were reactive and to a small extent. Proteins from the pelecypod *Pecten* had no measurable reactivity with the anti-*Busycon* serum.

The antiserum to the proteins of the pelecypod *Macoma nasuta* was strongly reactive with the proteins of *Pecten hericus,* but gave negative results with the proteins of the other pelecypods *Modiolus* and *Pododesmus.* Negative also were the results of tests performed with representatives from the other Classes in the Phylum.

The anti-*Modiolus* serum was slightly, and equally, cross-reactive with the proteins of the pelecypods *Pododesmus* and *Pecten.* Proteins from the pelecypod *Macoma* were not measurably reactive. Proteins from representatives from the Gastropoda, Cephalopoda, and Amphineura were likewise not measurably reactive.

Proteins from *Pecten hericus* and from *Pecten hindsii* each induced the formation of strikingly similar antisera. Good differentiation between species was obtained with both antisera when they were tested with the proteins of the two pectens. Cross-reactions were obtained between the proteins of the amphineurans *Cryptochiton* and *Katharina* and anti-*Pecten hericus,* and between *Cryptochiton* and anti-*Pecten hindsii.* Proteins of other amphineurans and proteins from the cephalopods and gastropods gave negative results when tested with these antisera.

The antiserum produced against the proteins of the pelecypod *Pododesmus macroschisma* was cross-reactive with the proteins of the pelecypod *Modiolus modiolus.* Results obtained with proteins from tests performed between this antiserum and the proteins from species representative of the other Classes of the Phylum Mollusca were negative.

The two antisera which were produced against the hemocyanins of the amphineuran *Cryptochiton stelleri* had differing specificities. Within the Amphineura, however, the sequence of relationship for cross-reacting proteins was the same for each antiserum. Proteins from *Katharina tunicata* had the strongest cross-reaction, followed by *Mopalia muscosa* and then *Tonicella lineata.* With one of the antisera, the proteins of the gastropod *Busycon* were more reactive than were the proteins of the cephalopods *Sepia* and *Octopus*; however, with this same antiserum, the proteins of the other gastropod, *Argobuccinum,* were negative. The other antiserum against *Cryptochiton* had a slight cross-reaction with the proteins of *Octopus* and no measurable reaction with the proteins of *Busycon.* The latter antiserum was also not measurably reactive with the proteins of the pelecypods.

The antiserum produced against the proteins of *Katharina tunicata* had its strongest cross-reaction with the proteins of *Cryptochiton. Mopalia* and *Tonicella* were next most reactive in that order. Of the species from other Classes the proteins of the cephalopod *Sepia officinalis* were most reactive. The proteins of the cephalopod *Octopus* were reactive to a lesser extent, as were the proteins of the gastropod *Busycon.* The proteins of the pelecypods had no detectable reactions with this antiserum.

Proteins from *Katharina tunicata* and *Cryptochiton stelleri* were approximately equally reactive with the anti-*Mopalia* sera and more so than were the proteins of *Tonicella lineata.* The proteins of *Octopus vulgaris* were slightly cross-reactive with this antiserum.

Reactions involving the antiserum produced against the proteins of *Tonicella lineata* were restricted to the proteins of the amphineurans. By means of the anti-

serum we were unable to distinguish effectively among the proteins of the species *Cryptochiton stelleri, Katharina tunicata,* and *Mopalia muscosa.*

Discussion

Only a few reports in the literature provide serological data on interclass relationships among the mollusks.

Galli-Valerio (1916) produced antisera against tissue-extracts of the pelecypod *Anodonta anatina* (Linnaeus). His only heterologous precipitin tests were with similar extracts from the gastropod *Limnaea stagnalis* (Linnaeus), which produced precipitates.

Erhardt (1931) produced antisera against the gastropods *Arion empericorum* Ferussac and *Limnaea stagnalis* (Linnaeus). The stronger anti-*Arion* serum cross-reacted well with the proteins of the other gastropods, but gave negative results in tests with the proteins of the amphineuran *Chiton marginatus* Pennant.

Makino (1934) employed precipitin, complement fixation and anaphylactic reactions with saline-extracted antigens from the pelecypods *Arca inflata* Reeve, *Cytherea meretrix* Linnaeus, *Ostrea gigas* Thunberg, *Paphia philippinarum* Adams and Reeve, the gastropods *Haliotis gigantea* Chemnitz, *Rapana thomasi* Crosse, *Turbo coronatus* (Gmelin) and the cephalopods *Sepiella japonica* Sasaki, and *Polypus* [= *Octopus*] *variabilis* Sasaki. Within each Class the cross-reactions between antigens and antibodies were strong. Cross-reactions between reagents which were representative of different Classes of mollusks were weak or doubtful, or gave negative results. His precipitin data were extensive and he judged that they indicated the gastropods resembled the pelecypods more than they did the cephalopods. This judgment was based on "ring test" data, the differential titers of which were within the experimental error of method, and generally on "plus" versus "plus-minus" reactions. In our opinion his data revealed no conclusive order of relationship among the three groups of mollusks.

Kuramoto (1933) made extensive interclass serological comparisons of representative species of pelecypods, gastropods and cephalopods. He produced antisera against the pelecypods *Ostrea gigas* Thunberg, *Corbicula japonica* Prime, *Cristaria plicata* Leach, *Cytherea meretrix* Linnaeus and *Tapes philippinarum* Adams and Reeve, the gastropods *Haliotis gigantea* Chemnitz, and *Viviparus japonicus* Martens and the cephalopods *Euprymna morsei* Verrill, and *Octopus membranaceus* Quoy. His precipitin ("ring-tests") and complement fixation data show, without question, a closer relationship between the gastropods and cephalopods than between either of these and the pelecypods. This work is singularly exceptional, in comparison to the findings of others, in that his results give strong interclass serological reactions which reciprocally verify one another.

Chestnut (1943) employed the turbidimetric analysis of precipitin reactions in his study of molluscan relationships. He produced antisera against the tissue-proteins of the pelecypods *Ostrea gigas* Thunberg and *Ostrea virginica* (Gmelin), the hemocyanins of the gastropods *Fasciolaria gigantia* Kiener, *Busycon perversum* Linnaeus, *Busycon caricum* (Gmelin), and *Aplysia protea* (Rang) and a cephalopod *Octopus* sp. The antisera against the pelecypod proteins were Class-specific, as were all but one of the antigastropod sera. A slight cross-reaction was obtained

between the anti-*Aplysia* serum and the proteins of the pelecypods. Other interclass cross-reactions were obtained with the anti-Octopus serum and the proteins from both the pelecypods and the gastropods. The gastropods were indicated to be slightly more closely related to the cephalopod than were the pelecypods.

Wilhelmi (1944) obtained reciprocal cross-reactions between antigens and antisera of the gastropod *Busycon caricum* (Gmelin) and the pelecypod *Pecten irradians* Lamarck.

Our serological data were inconclusive with respect to the establishment of a definite sequence of relationship among the four Classes of the Phylum Mollusca. The values for interclass relationships were generally low and approximately at the limit of the sensitivity of the serological methods which were employed. Multiple series of injections were given to induce the formation of powerful antisera. It was seemingly beyond the capabilities of the rabbits to produce antisera by means of which we could discriminate effectively among the cross-reacting proteins from the different Classes. Most of the proteins from species which were in the same Class as the homologous species were reactive with a given antiserum and readily distinguished from proteins from another Class. Were it not for the fact that the interclass data were chiefly at the limit of the ranges of the antisera, and close to the experimental error in the method, the negative results might otherwise have been significant as a means of distinguishing, in part, among the Classes. An inspection of the interclass data reveals several instances where the proteins of one species within a Class gave a measurable cross-reaction with an antiserum while the proteins of a second species from the same Class gave a negative result.

The pelecypod proteins gave fewer positive interclass reactions than did the proteins of the representatives of the other Classes. Moreover, most of the antisera produced against the proteins of the pelecypod species were more specific than the antisera produced against the proteins of species from other Classes. This pattern of cross-reactions for the pelecypods might have been due, in part, to the noncorrespondence of their proteins with the proteins of the species from the other Classes. The proteins of the pelecypods were in extracts obtained from homogenates of the whole bodies of organisms. Proteins from species in other Classes were the hemocyanins of the sera. The low cross-reactivity of the proteins of the amphineuran *Tonicella lineata,* which were obtained also as extracts and leachings from the bodies of the organisms, likewise might have been due to their lack of correspondence with the serum proteins of the other amphineurans. More difficult to comprehend are differences in cross-reactivity, in view of the generally aspecific nature of interclass serological reactions, of the hemocyanins from species in one Class with the antihemocyanin sera representative of another Class. The hemocyanins of the gastropod *Argobuccinum,* for example, failed to give a measurable cross-reaction with the anti-*Cryptochiton* serum, while the hemocyanins of the gastropod *Busycon* had seven per cent of measurable correspondence with the homologous reaction. The difference between the reactivities of *Argobuccinum* and *Busycon* exceeds the limits of the experimental error of the methods. Both of the antigens were collected and preserved in the same manner. Their places of origin were greatly different, but this fact should not be of importance if the same laws of inheritance and principles of systematics and evolution apply to the conservatively inherited proteins of the serum as apply to other essential characters of these

organisms. Lipoprotein-anti-lipoprotein reactions have been known (Boyden, 1936; Cumley, 1939; Wilhelmi, 1944) to account for otherwise unpredictable serological reactivity involving hemocyanins and other proteins from invertebrates. Lipoidal substances were not removed from the serum antigens which were used in our experiments. The presence of lipid materials in the serum of invertebrates might be a superficial trait, a consequence of the dietary habits of the animals, hence, under environmental control, and of trivial systematic importance in serological comparisons. Lipid materials bound to proteins as a consequence of genetically controlled syntheses are of unquestioned value in serological comparisons. The relatively aspecific nature of the fatty component of lipoproteins would provide the greatest usefulness in studies of serological correspondence among distantly related groups of animals. It was this reasoning which led us to use antigens which had not been defatted.

A critical evaluation of the interclass serological data in this paper, and in earlier reports (Erhardt, 1931; Kuramoto, 1933; Makino, 1934; Chestnut, 1943; and Wilhelmi, 1944), does not support Knight's suggestion (1952, p. 44) to merge the isopleuran (amphineuran) and anisopleuran (gastropod) mollusks into a single Class, the Gastropoda. By assuming the generally lowered interclass reactivities of the pelecypods (Table II) to be due to the lack of correspondence of their tissue-extract proteins with the hemocyanins of the sera of other groups and by recalling that most of the measurable interclass reactivities are within or near the experimental error of the method, all of the groups would stand distantly related, and approximately equally so. In this interpretation the four Classes Amphineura, Gastropoda, Pelecypoda, and Cephalopoda would be retained as commonly accepted.

Cross-reactions between serological reagents involving proteins from pelecypods, gastropods and cephalopods have been reported by Kuramoto (1933), Makino (1934), and Chestnut (1943). Each of these workers obtained results which indicated that the gastropods and cephalopods are more closely related to each other than either of them is to the pelecypods. Their data indicate, also, a closer relationship between pelecypods and gastropods, than between pelecypods and cephalopods. Our data yielded no conclusive information on the relative amounts of serological correspondence among the three groups.

Both of the anti-*Pecten* sera had reactions with the proteins of the amphineurans which were inconsistent with the other serological data in Table II. We can offer no explanation for the reactions. Subsequently-produced anti-*Pecten* sera gave no such cross-reactions with the proteins of the amphineurans. The reactions of the anti-*Macoma* serum are also unusual because of the strong cross-reaction of this serum with *Pecten* and the failure of the serum to react with the proteins of other pelecypods. These "anomalous" data are included in our results to illustrate the occasional occurrence of unverifiable serological reactions which are in all probability due to unusual responses of rabbits to the antigens being injected.

Summary

1. Serological comparisons were made with precipitins of proteins from molluscan species representative of the four Classes Amphineura, Gastropoda, Cephalopoda and Pelecypoda.

2. Interclass reactions were approximately at the limit of the sensitivity of the turbidimetric method which was employed.

3. Knight's proposal to include the amphineurans in the Class Gastropoda is not supported by the serological data. The analysis and integration of the serological data already in the literature, with the data in this work, provide additional evidence that the four groups should be retained as distinct Classes.

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